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MCA106 Fibrosarcoma Cells Transduced With Granulocyte/Macrophage Colony-Stimulating Factor Are Not Superior to the Wild-Type Cells in Suppressing the Growth of Hepatic Metastases

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Background and Objectives: Vaccination with cytokine gene-modified tumor cells augments the immune response against established tumors and protects against tumor challenges. In this study, we investigated the vaccine potential of GM-CSF–transduced MCA106 fibrosarcoma (MCA-GMCSF) cells in the C57BL/6 (B6) murine hepatic metastasis model.

Methods: Experimental mice received one to three weekly vaccines (subcutaneous/intramuscular, s.c./i.m.) of irradiated, parental, or GM-CSF–transduced MCA106 tumor cells. One week after the last immunization, hepatic metastases were established through the intrasplenic injection of live MCA106 parental (MCA106P) tumor cells. The animals were then sacrificed 3–4 weeks after surgery for evaluation of hepatic tumor burden.

Results: Based on in vivo experiments, both GM-CSF–modified and parental MCA106 tumor cell vaccines induced strong protection against hepatic tumor growth with grossly visible tumors rarely identified. This protection was evident even at a single vaccine dose of as low as 1×10^5 irradiated cells. Unimmunized control mice, on the other hand, consistently developed substantial hepatic tumors. Cytotoxicity assays on splenocytes (cultured in vitro for 4–5 days) showed that both groups of vaccinated mice developed strong tumor-specific cytotoxic T-lymphocyte (CTL) responses. Immunohistochemical analysis of injection sites showed infiltration of dendritic cells (DCs) and macrophages into subcutaneously injected MCA-GMCSF cells. Mostly macrophages, however, were seen at the injection site of MCA106P cells. Furthermore, the MCA106P cells expressed high levels of MHC class I antigens and the level of expression was not significantly altered by transduction with the GM-CSF gene. The high expression of MHC class I antigens probably contributed to the strong immunogenicity of the MCA106P cell vaccine.

Conclusions: This study demonstrates that MCA106 parental cells are as effective as the GM-CSF–transduced cells in suppressing the growth of hepatic metastases. The cellular immune responses induced by these two vaccines, however, are probably mediated by different subsets of host

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effector cells. These results have important implications for the use of GM-CSF–transduced cell vaccines in the immunotherapy of tumors that have the propensity to metastasize through the lymphatic channels and the circulatory system. *J. Surg. Oncol. 1999;71:36–45.*

KEY WORDS: GM-CSF; MCA106 fibrosarcoma; immunotherapy; tumor metastases; dendritic cells; T-cells

INTRODUCTION

Treatment of cancers through the augmentation of the host immune response remains a desirable and intensely investigated area. Host antitumor immunity can be enhanced via immunization with tumor cells, especially those genetically engineered to produce cytokines such as interleukin IL-2 [1–6], IL-4 [7], IL-6 [8], IFN-γ [5,6,9,10], or granulocyte/macrophage colony-stimulation factor (GM-CSF) [11,12]. With the introduction of specialized packaging cell lines that allowed the production of high titers of replication-defective recombinant retrovirus, the process of transducing tumor cells with cytokine genes has become simpler and more efficient [13]. This technology has permitted the use of cytokine gene therapy in clinical trials. By comparing the vaccine potential of cytokine gene-transduced tumor cells, Dranoff et al. [11] found that GM-CSF is the most potent cytokine for stimulating long-lasting antitumor immunity in the B16 melanoma animal model. The strong immunotherapeutic effect of GM-CSF is attributed to its ability to induce the proliferation and differentiation of multipotential bone marrow progenitor cells into antigen presenting cells [14,15]. GM-CSF has also been implicated as a cytokine necessary for the generation of dendritic cells (DCs) from peripheral blood [16–19]. In addition to stimulating secondary responses, DCs are efficient at initiating primary cellular responses [19,20]. The ability of DCs to stimulate T-cell responses against tumor cells is enhanced by GM-CSF [19,20]. The strong antitumor effect of GM-CSF in the B16 melanoma study [11] was thought to be mediated by DCs that were recruited to tumor sites by the released GM-CSF [11,12].

GM-CSF–transduced tumor cells have been utilized for the immunization of patients with highly metastatic tumors such as melanoma and renal cell carcinoma [21,22]. The results of these clinical trials were not encouraging, despite the success encountered with GM-CSF in animal experiments. Ellem et al. [21] found only partial and temporary clinical benefits using GM-CSF–transduced autologous melanoma vaccine in a patient with progressive and widely disseminated disease. Furthermore, in the clinical trial by Simon et al. [22], only 1 of 16 patients showed partial response to GM-CSF gene-modified autologous renal cell carcinoma vaccine. So far, the number of patients who have benefited from the GM-CSF–transduced tumor cell vaccine is small and more work is required to evaluate the efficacy of this treatment modality.

In the current study, we evaluated the vaccine potential of MCA106P and GM-CSF–transduced cells in C57BL/6 mice using a hepatic metastasis model previously established in our laboratory. We examined the effect of GM-CSF–modified MCA106 cells in protecting the host against the wild-type MCA106 cells. The mechanism of protection by the parental and the GM-CSF–modified cell vaccines was also investigated.

MATERIALS AND METHODS

Animals

The C57BL/6 mice (5–7 weeks old, female) were obtained from Charles River Laboratories (Raleigh, NC) and were housed at the Veteran's Administration Medical Center (VAMC) animal care facilities in Durham, NC. All studies were conducted in accordance with the animal care policy of Duke University Animal Care and Use Committee. The VAMC animal facilities and Duke University are approved by the American Association for Accreditation of Laboratory Animal Care (AAALAC).

Tumor Cells

The MCA106 fibrosarcoma cell is a methylcholanthrene-induced fibrosarcoma of C57BL/6 origin (H-2^b background). MCA106 fibrosarcoma cells were plated in T75 flasks (Costar, Cambridge, MA) as monolayers in Minimum Essential Medium (MEM, Gibco, Grand Island, NY), supplemented with 5% fetal bovine serum (FBS, Hyclone Labs, Logan, UT), penicillin (100 units/ml), streptomycin (100 μg/ml), and 2-mM L-glutamine. The monolayers were propagated by trypsinization as required. These tumor cells were transduced with the murine GM-CSF retroviral vector and were cultured in MEM, which was supplemented with 500 μg/ml of the selection agent G418 (Geneticin, Gibco). Yac-1 cells (H-2^b background, and NK-sensitive target) and P815 (H-2^d background, a mastocytoma) were used as controls in cytotoxicity assays and were maintained in RPMI medium supplemented with 10% FBS, 2-mM L-glutamine, penicillin (100 units/ml), streptomycin (100 μg/ml), and gentamicin (50 μg/ml). Tumor cells were purchased from American Type Culture Collection (ATCC, Rock-

ville, MD) or obtained from our collaborators at Viagene (San Diego, CA).

Transduction of MCA106 Tumor Cells

A retroviral vector carrying the gene for the murine GM-CSF cytokine (plus a neomycin selection marker) was provided by Dr. T. Fong (Chiron Viagene, San Diego, CA). MCA106 parental tumor cells (1×10^6) were plated in T25 flasks (Costar) in MEM containing polybrene (4 μg/ml, Sigma, St. Louis, MO). An aliquot of the GM-CSF vector was added to the cells at a multiplicity of infection of 10:1. After 4 hr, the medium was aspirated and replaced by fresh medium containing another aliquot of the vector. The cells were then incubated with the vector for 18 hr at 37°C. Next, cells were placed in a selection medium that contained G418 (500 μg/ml in MEM-10% FBS). Colonies that survived the selection period were expanded and cryopreserved. A large stock of the transduced cells was frozen before the beginning of animal experiments. To ensure the use of uniform cells in the study, the transduced cells were propagated in culture for only 1 month, after which time a new vial of the frozen stock was thawed for experiments. Additionally, cytokine secretion was assessed periodically by ELISA.

Detection of Cytokine Production

The amount of GM-CSF produced by the MCA106-transduced tumor cells was quantified using an ELISA kit (Endogen, Woburn, MA). A total of 1×10^6 GM-CSF–transduced MCA106 cells were plated for 48 hr in 10-ml medium in a T25 flask. The medium was collected following the culturing period and was then analyzed using an ELISA kit, as per manufacturer's protocol. Supernatant from MCA106 parental cells was tested in the same ELISA assay.

Immunization With MCA106 Tumor Cell Vaccine

Parental and GM-CSF–modified MCA106 cells were removed from culture flasks by trypsin on the day of injection. Cells were then washed twice in Hanks' balanced salt solution (HBSS), resuspended in HBSS that contained 2% human serum albumin (HSA), and counted. Prior to inoculation, the cells were irradiated with 10,000 rad of γ-radiation at a rate of 2,247 rad/min using a Mark I ^{137}Cs irradiator (J.L. Sheperd and Associates, Glendale, CA). Each mouse was injected s.c./i.m. in the right hind leg with irradiated parental or transduced MCA106 tumor cells. Vaccine doses ranged from 1×10^5 to 1×10^6 irradiated tumor cells. The entire process was repeated 7 days later for mice receiving multiple immunizations.

Cytotoxicity Assays

Seven days after the last vaccine, spleens were harvested from mice immunized with irradiated parental, or GM-CSF–transduced cells. Spleens were pressed through a sterile steel mesh into a petri dish and suspended in HBSS. Red blood cells were depleted by incubating splenocytes in 0.84% ammonium chloride (pH 7.5) for 15 min at 37°C. Splenocytes were then washed twice with HBSS and resuspended in RPMI medium that was supplemented with 10% FBS, penicillin, streptomycin, L-glutamine, IL-2 (10 units/ml, kindly provided by Chiron Viagene), and 2β-mercaptoethanol (2×10^{-5} M, Gibco). The isolated spleen cells were cultured on a feeder layer of irradiated MCA106P cells at a ratio of 200:1 (splenocytes:tumor cells) and incubated in T25 flasks in upright position at 37°C with 5% CO_2.

Cytotoxicity assays were performed on day 4 or 5 of in vitro culturing following a standard chromium release protocol. Briefly, target cells were suspended in 1 ml of culture medium that contained 100 μCi of ^{51}Cr (New England Nuclear, Boston, MA), and incubated for 1 hr at 37°C. After three washes in HBSS, the targets were resuspended in RPMI-10% FBS medium at a concentration of 5×10^4 cells/ml. The effector splenocytes were serially diluted in 96-well plates (100 μl/well) at effector-to-target ratios varying between 100:1 and 12.5:1. Washed target cells were added to these wells and the final volume was adjusted to 200 μl/well. The plates were centrifuged at 200 × g for 5 min and incubated at 37°C for 6 hr. The supernatant was collected using harvesting frames (Skatron Harvesting System, Skatron, Sterling, VA) and counted in a gamma counter (Packard Instrument, Downers Grove, IL). Target cells in RPMI medium or 1-M HCl represented the spontaneous and maximum releases, respectively. Percent specific lysis was calculated as follows: [(mean experimental cpm − spontaneous release)/(maximum release − spontaneous release)] × 100.

Fluorescence-Activated Cell Sorter (FACS) Analysis

Tumor cells and splenocytes were analyzed for the expression of cell surface antigens. Tumor cells were incubated at 4°C for 30 min with monoclonal antibodies (mAB) directed against H-2K^b, H-2D^b, and I-A^b MHC cell surface antigens (hybridomas 28-13-3s, 28-14-8s, and 28-16-8s specific for these MHC antigens, respectively, were obtained from ATCC). Splenocytes were incubated under the same conditions with mABs against CD4 and CD8 (fluorescein-conjugated, Caltag Laboratories, Burlingame, CA) surface markers. Also, NK cells were detected using the PK136 mAB (ATCC). Cells were washed twice with phosphate-buffered saline (PBS) containing 2% FBS and 0.02% sodium azide (Sigma). Fluorescein isothiocyanate-conjugated goat antimouse

Fab′2 secondary antibody (TAGO, Burlingame, CA) was then added and incubated with the cells (which reacted with unlabeled primary antibody) at 4°C for another 30 min. Next, cells were washed twice and fixed in PBS with 1% formalin. The samples were analyzed using a FACS flow cytometer (Becton Dickinson, Sunnyvale, CA).

Generation of Subcutaneous Tumors and Immunohistochemistry

Subcutaneous tumors were generated to examine the host cellular response at tumor sites. Mice were injected in the hind footpad with 5×10^5 viable (unirradiated) MCA106P or MCA-GMCSF tumor cells suspended in 50-μl HBSS. These animals were sacrificed at different time points after the inoculation. Tumor tissue was dissected from the footpad, snap-frozen in liquid nitrogen, and stored at −70°C until slide preparation. The frozen tissue was first embedded in TECK OCT compound (Miles, Elkhart, IN) and then sectioned into 5-μm slices using a microtome. Tissue sections were air-dried, fixed in acetone for 10 min, and then treated with methanol plus 0.37% H_2O_2 for 30 min to block endogenous peroxidase activity. Next, slides were washed and incubated in PBS containing 1% BSA and 5% horse serum for another 30 min to block nonspecific binding sites. Biotinylated mAB against CD3e, CD4, CD8b.2, CD11b (mac-1), and CD86 (Pharmingen, diluted 1:25), were added to tissue sections for 45 min. Next, slides were washed and incubated with horseradish peroxidase (HRP)-conjugated streptavidin (diluted 1:300, Zymed Labs, San Francisco, CA) for another 30 min. Antibody staining was revealed with the substrate diaminobenzidine (DAB, Sigma), diluted to a concentration of 0.5 mg/ml in Tris buffer (0.05 M, pH 7.6), and activated using 0.5 μl/ml of 37% H_2O_2. The sections were then counterstained with hematoxylin.

Generation of Hepatic Tumor Metastases

Both unimmunized and immunized mice were challenged with MCA106 parental tumor cells intrasplenically. Surgical procedures were performed under sterile conditions. After anesthetizing the mice individually using halothane vapor, their abdomens were prepared for surgery with povidone-iodine solution. Initial incision was made at the left subcoastal space into the peritoneum. The spleen was identified and delivered out through the abdominal wall. Each spleen was injected with 3×10^5 MCA106P tumor cells in 200-μl HBSS over a period of 1 min to ensure adequate absorption (1×10^6 MCA106P cells were used in some experiments). After inoculation, the splenic pedicle was ligated with a 3-0 silk suture and the spleen was excised. Peritoneal and skin incisions were then closed with 2-0 silk running sutures. Mice were allowed to recover in a warmed environment and then returned to their respective cages under close supervision for the ensuing weeks. These mice were sacrificed 3–4 weeks after surgery. The abdomens were explored and livers were removed and examined for tumor metastases. Tumor burden was assessed based on liver weight and tumor mass (identified by gross examination).

TABLE I. Expression of MHC Antigens on MCA106P and MCA-GMCSF Tumor Cells*

Cells	Baseline	K^b	D^b	I-A^b
MCA106P	4.63	33.03	39.87	6.82
MCA-GMCSF	3.08	17.44	22.58	5.33

*Cells were reacted with hybridoma supernatants specific for the indicated antigens followed by a FITC-labeled secondary antibody. Values in the table represent the mean fluorescence channel number for the total cell population. Cells reacted with FITC-labeled secondary antibody without hybridoma supernatant are shown as baseline values.

Statistical Analysis

Each in vitro cytotoxicity experiment was performed at least twice, and the result of a representative experiment is shown. Results of in vivo experiments for hepatic metastases were pooled. Differences in tumor weights between mice groups were assessed using Student *t*-test. A P value of less than 0.05 was considered significant.

RESULTS

MCA106 Parental Cells Are Readily Transducible With Murine GM-CSF Gene

The transduced cells survived the G418 selection and produced murine GM-CSF at a rate of approximately 48 ng/day/10^6 cells as estimated by ELISA (which was performed three times during the study). The MCA106P tumor cells did not secrete GM-CSF when tested along with the transduced cells in the same assay. The transduced cells continued to secrete GM-CSF after several weeks of in vitro culturing in a medium containing 500 μg/ml of G418. The GM-CSF–modified cells were not different from the parental cells in the in vitro growth characteristics. There were some minor differences, however, between MCA106 parental and the GM-CSF–transduced cells in the level of expression of the MHC molecules (Table I). Both tumor cell types expressed high levels of MHC class I molecules as indicated by the mean fluorescence intensity for K^b and D^b antigens. The MCA106P tumor cells, however, consistently displayed higher levels of K^b and D^b surface antigens as compared to the MCA-GMCSF–modified cells. Both cell types displayed low levels of MHC class II (I-A^b) antigens (Table I).

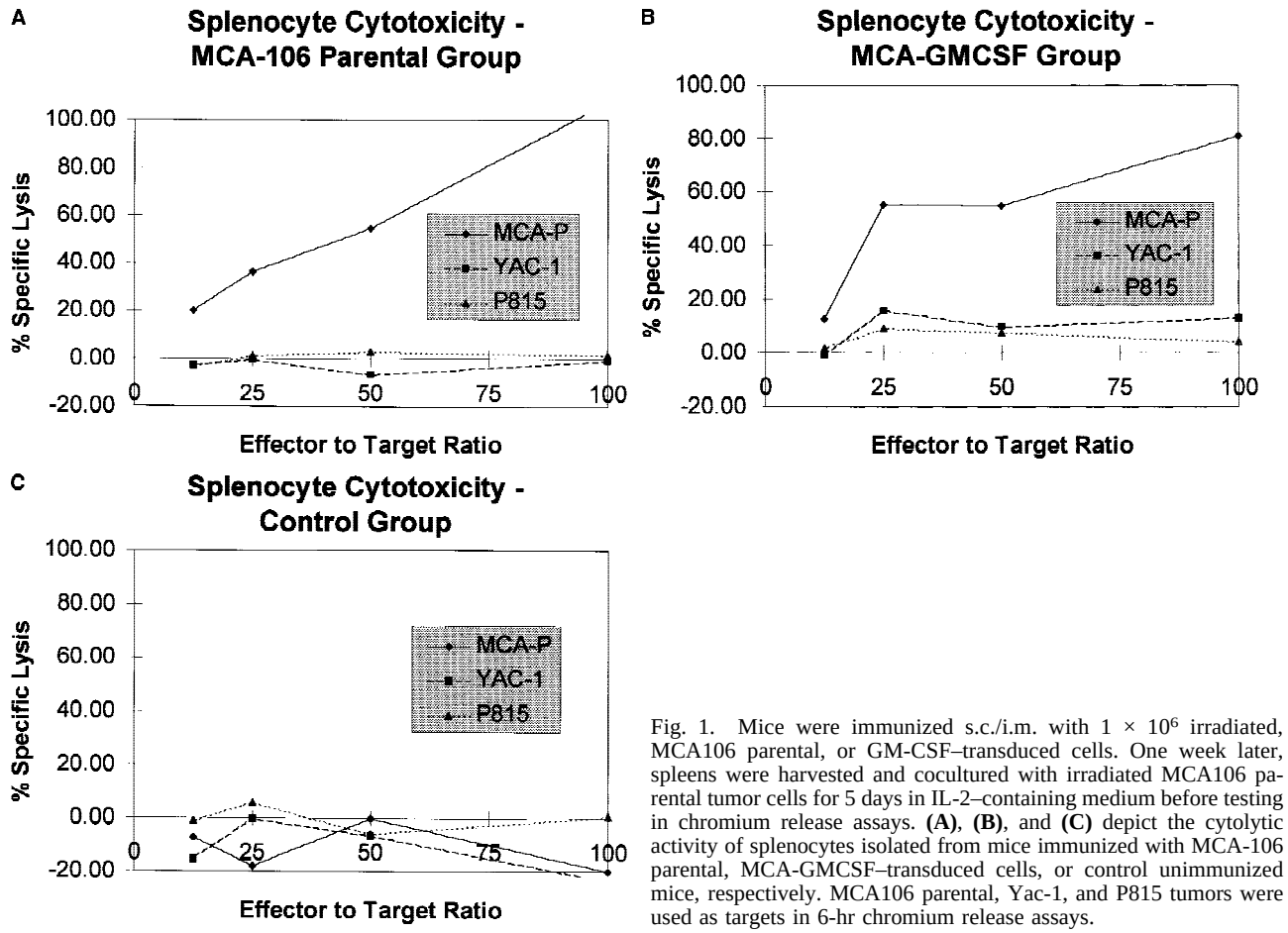

Fig. 1. Mice were immunized s.c./i.m. with 1×10^6 irradiated, MCA106 parental, or GM-CSF–transduced cells. One week later, spleens were harvested and cocultured with irradiated MCA106 parental tumor cells for 5 days in IL-2–containing medium before testing in chromium release assays. **(A)**, **(B)**, and **(C)** depict the cytolytic activity of splenocytes isolated from mice immunized with MCA-106 parental, MCA-GMCSF–transduced cells, or control unimmunized mice, respectively. MCA106 parental, Yac-1, and P815 tumors were used as targets in 6-hr chromium release assays.

Generation of Antitumor Cytotoxic T-Lymphocytes (CTLs) in Mice Immunized With Either Parental or GM-CSF–Modified MCA106 Cells

The development of MCA106-specific cellular response in mice immunized with either parental or GM-CSF–transduced tumor cells was tested by cytotoxicity assays. Seven days after a single vaccination with 1×10^6 irradiated cells, splenocytes from the MCA106P and MCA-GMCSF groups were isolated and cultured for 4–5 days in IL-2–containing medium. Both groups showed significant lytic activity against MCA106 parental target cells (Fig. 1A and B). No cytotoxic activity was found in splenocytes of control unimmunized mice (Fig. 1C). The lytic activity closely paralleled the effector-to-target (E:T) ratios tested in these assays. Strong specific lysis of approximately 100% (at E:T ratio of 100:1) was detected in some assays for both the MCA106P and MCA-GMCSF–immunized groups. The lysis was tumor-specific as suggested by the lack of killing of P815 or Yac-1 cells in vaccinated groups (Fig. 1A and B). Similar results were detected in mice immunized with two injections of 1×10^6 irradiated cells (data not shown), suggesting that the GM-CSF–transduced, as well as the parental cells, are capable of inducing CTL activity in mice.

Splenocytes From Immunized and Control Mice Display Similar Phenotype

Splenocytes were cocultured in vitro for 4–5 days with MCA106 parental cells in RPMI medium + IL-2, and then tested in cytotoxicity assays and phenotyped. FACS analysis was performed with antibodies specific for CD4, CD8, and NK (PK136) cells. The phenotype was not significantly different among the three groups in terms of the mean fluorescence intensity based on FACS analysis (Table II). This phenotype suggests that the significant increase in lytic activity for splenocytes derived from immunized vs. control mice is probably due to the high lytic activity on a per cell basis rather than increases in the percentage of $CD8^+$ or NK cells.

Macrophages and DCs Are Detected in MCA106 Tumors

Subcutaneous tumors generated from either MCA106P or MCA-GMCSF–transduced cells were palpable by day 7 and became necrotic after 25 days. Tissue sections from both tumors showed a sparse inflammatory infiltrate on day 7 based on hematoxylin and eosin (H&E) staining (data not shown). Immunohistochemical staining with CD11b mAB detected large numbers of macro-

TABLE II. Immunofluorescence Analysis of Splenocytes*

Mice	Baseline	CD4	CD8	PK136
Control[a]	4.51	10.96	16.41	39.12
MCA106P[b]	4.84	9.74	11.21	29.57
MCA-GMCSF[b]	4.78	9.26	14.43	34.02

*Values in the table represent the mean fluorescence channel number for the total cell population. The results are those obtained in one assay. The experiment was repeated twice. Splenocytes were derived from unimmunized mice or mice vaccinated once with either MCA106P or MCA-GMCSF cells. Splenocytes were cultured in vitro for 4 days in RPMI + IL-2 and then reacted with monoclonal antibodies against the indicated cell surface markers for FACS analysis. Cells reacted only with secondary antibodies are shown as baseline values.
[a]Splenocytes derived from unvaccinated mice served as controls.
[b]Mice were immunized with the indicated tumors before isolation of splenocytes.

TABLE III. Immunohistochemical Examination of Subcutaneous Tumors*

Tumor source	CD11b	CD3	CD4	CD8	CD86
MCA-106 parental					
Day 7	+++	++	+/−	+/−	+
Day 12	+++	+/−	+/−	−	+/−
MCA-GMCSF					
Day 7	+++	+	−	+/−	++
Day 12	+++	++	+/−	+	++

*Tumors were generated in the hind footpads of mice via subcutaneous injections of 5×10^5 MCA106 parental or MCA-GMCSF tumor cells suspended in 50-μl HBSS. Growing tumors were removed at day 7 and day 12 and immediately snap-frozen and sectioned for immunohistochemical staining. Tissues were first incubated with biotinylated monoclonal antibodies directed against CD11b (macrophages), CD3, CD4, CD8, and CD86 (B7-2), followed by reaction with HRP-labeled streptavidin. Reactions were detected with a DAB-H_2O_2 substrate. Slides were evaluated with scores ranging from − to +++, where − indicated no reaction and +++ represented a strong reaction.

phages in tumors developed from either MCA106P or MCA-GMCSF cells (Table III). Tumors derived from GM-CSF–modified cells contained a large number of CD86-positive cells as compared to that derived from MCA106P cells. These CD86 positive cells are most likely DCs. The amount of T-cells recruited to tumor sites in both groups was not significant compared to macrophages and DCs (Table III). The immunohistology provides insight into the host cellular response, which consisted of macrophages and DCs for the GM-CSF-secreting tumor, and mainly macrophages for the MCA106P tumor.

Vaccination With MCA106P or MCA-GMCSF–Modified Cells Provides Protection Against Hepatic Metastases

Vaccination with either MCA106P or MCA-GMCSF–irradiated tumor cells resulted in strong protection against hepatic metastases in the in vivo mouse model (Figs. 2 and 3, Table IV). Mice immunized with one injection (1×10^6 cells/mouse) were completely protected from developing hepatic tumors when challenged with 3×10^5 parental tumor cells intrasplenically (Fig. 3B and C). Unimmunized control mice (Figs. 2 and 3A) exhibited massive hepatic tumors. Figure 3 shows that one vaccine with either the parental or the GM-CSF–transduced cells is sufficient to elicit pronounced protection against tumor challenge. The induced host response was strong and additional vaccinations were of no benefit at this vaccine dose (Fig. 2, Table IV). The mean liver weight in the control group was 2.56 g, as compared to liver weights of less than or equal to 1.35 g in the vaccinated groups (Table IV).

In addition, we analyzed further the efficacy of these two tumor cell vaccines (parental vs. GM-CSF–modified) by altering the dose used for vaccination and that used for tumor challenge. At a vaccine dose of as low as 1×10^5 cells per mouse, animals immunized with either the parental or the modified cells were still protected against hepatic tumors (Table V). Most importantly, we have increased tumor challenge to 1×10^6 live parental cells in this experiment (compared to 3×10^5 cells used in the experiments shown in Figures 2 and 3 and Table IV). The increase in the challenging dose resulted in high operative mortality without increases in hepatic tumor burden (Table V). Only one of eight mice in the MCA-GMCSF–vaccinated group developed grossly visible tumors at this low immunization/high challenging dose protocol (Table V). In this experiment, the mean liver weights were 1.72 g for the control group compared with 1.30 and 1.59 g for the MCA106P– and

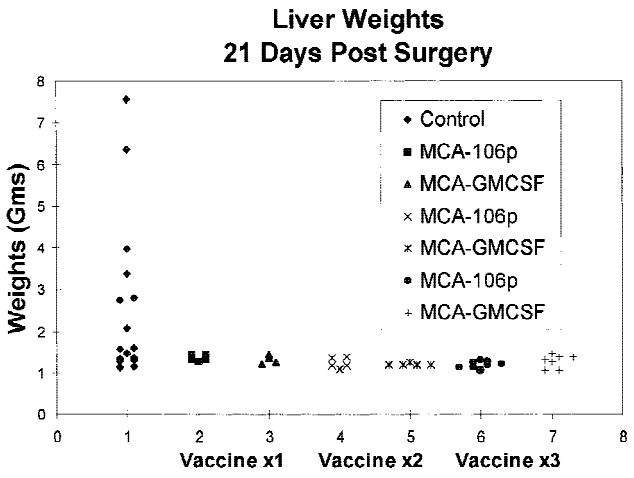

Fig. 2. B6 mice were immunized with one, two, or three vaccines. Each vaccine consisted of a weekly injection of 1×10^6 lethally irradiated MCA106 parental or GM-CSF–modified cells. These mice were then challenged with an intrasplenic injection of 3×10^5 live MCA106 parental cells to generate hepatic metastases. Mice were sacrificed 21 days postsurgery. Livers were isolated and weighted to assess tumor burden.

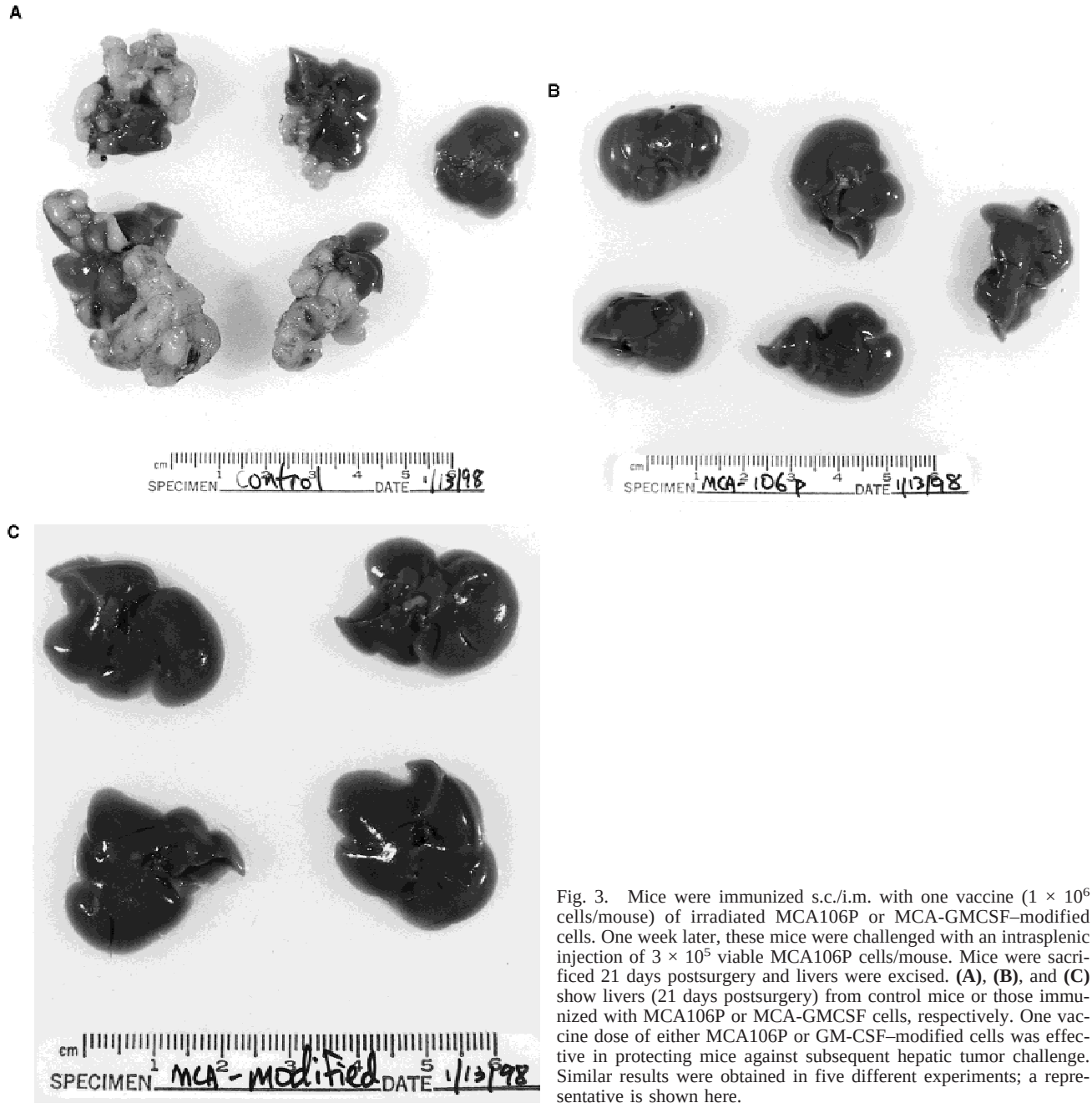

Fig. 3. Mice were immunized s.c./i.m. with one vaccine (1×10^6 cells/mouse) of irradiated MCA106P or MCA-GMCSF–modified cells. One week later, these mice were challenged with an intrasplenic injection of 3×10^5 viable MCA106P cells/mouse. Mice were sacrificed 21 days postsurgery and livers were excised. **(A)**, **(B)**, and **(C)** show livers (21 days postsurgery) from control mice or those immunized with MCA106P or MCA-GMCSF cells, respectively. One vaccine dose of either MCA106P or GM-CSF–modified cells was effective in protecting mice against subsequent hepatic tumor challenge. Similar results were obtained in five different experiments; a representative is shown here.

the MCA-GMCSF–vaccinated groups, respectively. The mean liver weight was not statistically different between the control and the MCA-GMCSF group due to an outlier mouse that developed gross tumors in the MCA-GMCSF group (Table V). These experiments demonstrate clearly that the MCA106 parental tumor cell vaccine is as effective as the GM-CSF–modified vaccine in suppressing the growth of hepatic metastases. The data show that a small vaccine dose of as low as 1×10^5 MCA106P or GM-CSF–transduced cells is sufficient to induce protection against hepatic tumors.

DISCUSSION

Gene therapy has been in the forefront of research because of its potential application in the management of many human diseases, including highly metastatic cancers [23–25]. Tumor cell vaccines can stimulate tumor-specific immune reactions, but the induced responses are generally inadequate to mediate tumor rejection [26]. The weak antitumor effect of cell vaccines is due to a number of reasons, including low expression of MHC molecules on tumors, defects in antigen processing, and

TABLE IV. Suppression of the Growth of Hepatic Metastases in Vaccinated Mice*

Group	Control	MCA106P (1 Vac)	MCA-GMCSF (1 Vac)	MCA106P (2 Vac)	MCA-GMCSF (2 Vac)	MCA106P (3 Vac)	MCA-GMCSF (3 Vac)
Sample size	16	5	4	5	5	8	7
Mean liver weight	2.56	1.35	1.31	1.23	1.20	1.19	1.27
Standard deviation	1.92	0.08	0.10	0.13	0.03	0.09	0.16
P value[a]		0.01	0.01	0.008	0.006	0.006	0.009

*Mice were unimmunized (control) or immunized with 1–3 weekly injections of parental or GM-CSF–modified MCA106 cells. The mean weight of livers (at day 21 after intrasplenic injection of MCA106P tumor cells) and standard deviations are shown. *P* values were calculated using a one-tailed Student *t*-test assuming unequal variances on comparing different vaccinated groups to control unimmunized mice. Vac denotes vaccination.

[a]Comparisons between MCA106P and MCA-GMCSF are statistically nonsignificant at the 0.05 level for each vaccine injection. Values are therefore not recorded in this table.

reduction of costimulatory signals on vaccine cells [27–30]. The interest in tumor cell vaccines was renewed after the emergence of techniques that enhance the immunogenicity of tumor cells. Transduction of tumor cells with genes for cytokines [1–12], costimulatory, or antigen presenting molecules [31,32] increases the therapeutic potential of vaccines. Poorly immunogenic murine tumors can be genetically engineered to release cytokines, a modification that promotes their immunogenic properties and reduces their tumorigenicity [11,33]. Transduction of tumor cells with cytokines such as IFN-γ has been shown to upregulate the expression of MHC molecules [5,34,35].

The potential to improve the immunotherapeutic function of tumor cell vaccines was extensively examined in the poorly immunogenic B16 murine melanoma model [29,33,36,37]. When transduced with the GM-CSF gene, B16 melanoma cells became better stimulators of the host immune system [11]. The recruitment of antigen presenting cells to tumor sites was thought to be responsible for the improved immunogenicity of the GM-CSF–transduced B16 cell vaccine [12]. Professional antigen presenting cells such as DCs are known to engulf and process tumor antigens stimulating tumor-specific T-cell responses [38–40].

TABLE V. Effect of a Small Vaccine Dose (1×10^5 Cells) on Protection Against Hepatic Metastases*

Group	Control	MCA106P	MCA-GMCSF
Sample size	10	7	8
Mean liver weight	1.72	1.30	1.59
Standard deviation	0.39	0.13	0.65
P value[a]		0.004	0.306[b]

*Mice were immunized with one injection of 1×10^5 irradiated parental or GM-CSF–modified MCA106 cells. One week later, these mice were challenged by intrasplenic injection of 1×10^6 live MCA106 parental cells. Mice were sacrificed 21 days postsurgery and livers were examined. Mean liver weights and standard deviations are shown. *P* values, calculated based on a one-tailed Student *t*-test, are shown, reflecting comparisons between vaccinated groups and control mice.

[a]Comparison of the mean liver weights between MCA106P and MCA-GMCSF is statistically nonsignificant at the 0.05 level using a one-tailed Student *t*-test assuming unequal variances.

[b]One of eight mice immunized with GM-CSF–modified cells developed large tumor burden, reducing the significance of the *P* value in this experiment.

The murine MCA106 fibrosarcoma cells express high levels of MHC class I antigens [41]. Transduction of MCA106 with the GM-CSF gene did not alter the surface phenotype, except for a minor reduction in the expression of class I MHC antigens. This is in agreement with Ciotti et al. [42], who reported the reduction of MHC class I expression in a modified melanoma cell line after GM-CSF secretion had attained high levels. MHC class I molecules are known to present tumor antigens to $CD8^+$ T-cells, which can stimulate cytolytic responses [28]. The high antitumor activity of the MCA106 parental tumor cells is most likely due to the expression of large amounts of MHC class I molecules on the surface [34,41,43,44]. We examined changes in the immunogenicity of MCA106 cells following transduction with the GM-CSF/retroviral construct. Indeed, GM-CSF enhances host immunity by increasing the presentation of tumor antigens to T-cells via dendritic cells [45]. In our study, however, a significant number of macrophages was demonstrated, based on histology, in the s.c. tumors generated from both MCA106P and MCA-GMCSF cells. Several studies have shown that macrophages are excellent antigen presenting cells, especially in tumors with high expression of MHC class I molecules [46,47]. Our data suggest that the role of macrophages in tumor rejection was as effective as that of DCs. Recent studies have also shown that monocyte-derived macrophages were comparable to dendritic cells in the presentation of class I-restricted peptides in vitro [48]. Although both tumor cell vaccines (MCA106P and MCA-GMCSF) were comparable in function, a different mechanism may be involved in mediating tumor rejection in each case.

Although several clinical trials are underway, preliminary results using cytokine-transduced cell vaccines have not generated favorable responses comparable to that

seen in animal studies [22,26]. The effectiveness of the vaccine in the murine models does not necessarily guarantee its success in humans, and results must be interpreted accordingly [26]. Hock et al. [49] studied the use of cytokine-transduced cell vaccines in a murine plasmacytoma model. They found that transduction of tumor cells with IL-2, IL-4, IL-7, TNF, or γ-INF cytokines did not improve the efficacy of the vaccines over that produced by vaccination with parental cells admixed with the classical adjuvant *Corynebacterium parvum* [49].

Transduction of tumor cells with a single cytokine may not enhance the immunogenicity of the vaccine. Several groups are examining methods to upregulate the immune response effectively through transduction with a combination of cytokines or the use of alternative adjunctive treatments. The combinatorial use of a biological response modifier such as OK-432, bacillus Calmette-Guérin (BCG), or *Corynebacterium parvum* enhances the antitumor activity induced by GM-CSF [50,51]. Some groups have intensified the vaccine potential of GM-CSF–modified cells by combining it with other cytokines (such as IL-4) or coupling it with tumor-specific monoclonal antibodies [52,53]. With these options available, the use of gene therapy in human cancers remains a viable approach.

CONCLUSIONS

This study demonstrates that tumor cells that express high levels of MHC class I antigens on the surface may provide effective vaccines for immunotherapy. Immunization with parental unmodified MCA106 tumor cells induces a strong CTL response and confers protection against hepatic metastases. This vaccine was effective even at a single dose of as low as 1×10^5 cells/mouse. The MCA106P cells were as effective as the GM-CSF–modified cells in this vaccine model. However, the GM-CSF–modified cells may be more efficient than the MCA106P cells in the therapeutic setting for the eradication of already established tumors. This subject has not yet been examined in our laboratory. In summary, tumor cells should be carefully studied in vitro and in vivo prior to genetic manipulation with cytokines.

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